Environmental Health Perspectives
Vol. 45, pp. 51-56, 1982

Mitochondrial Toxicity of Phthalate Esters

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The effects of mono- and dibutyl phthalate and mono- and di(2-ethylhexyl) phthalate on energy-dependent K^+ uptake, respiration rates, and succinate cytochrome c reductase activities of isolated rat liver mitochondria were evaluated. The energy-coupling processes, active K^+ transport and oxidative phosphorylation, were affected most by di-*n*-butyl phthalate and mono(2-ethylhexyl) phthalate. Mono-*n*-butyl phthalate had a moderate effect on energy coupling and di(2-ethylhexyl) phthalate had no apparent effect. The potency of inhibition of succinate cytochrome c reductase activity was mono(2-ethylhexyl) phthalate > di-*n*-butyl phthalate > mono-*n*-butyl phthalate = di(2-ethylhexyl) phthalate. It is concluded that phthalate esters affect mitochondrial activities by altering the permeability properties of the inner membrane and by inhibiting succinate dehydrogenase activity.

Introduction

The widespread distribution of phthalate ester plasticizers in the environment and the detection of significant quantities of di(2-ethylhexyl) phthalate (DEHP) in human blood stored in poly(vinyl chloride) bags (*1*) have led to concern about the possible biological effects of these compounds. An important observation is found in the NCI/NTP bioassay report (*2*) that DEHP is a hepatocarcinogen in $B6C3F_1$ mice and Fischer 344 rats. Hepatic changes have been reported after both oral and intraperitoneal administration of DEHP. One specific *in vivo* effect that has been identified is the decrease in mitochondrial succinate dehydrogenase activity (*3*, *4*). In addition, swollen liver mitochondria were observed in rats (*3*) after oral administration of DEHP or mono(2-ethylhexyl) phthalate (MEHP). Studies *in vitro* have revealed that the oxidative and phosphorylation processes of rat liver mitochondria are impaired by phthalate esters (*5-7*). Takahashi (*6*) studied the effects of dialkyl and monoalkyl phthalate esters on mitochondrial respiration rates with succinate as the oxidizable substrate. Respiratory control ratios (which are indicative of coupling between oxidative and phosphorylation activities) of isolated rat liver mitochondria decreased most with dialkyl phthalates that contain alkyl chains of three or four carbons, i.e., di-*n*-propyl and di-*n*-butyl phthalates. DEHP had no apparent effect on respiratory control. For the monoalkyl phthalates, respiratory control ratios decreased as the alkyl chain was increased up to seven carbons. MEHP was one of the most effective phthalate esters in reducing the respiratory control ratio.

We sought to explain the mechanism(s) by which phthalate esters interfere with mitochondrial activities. A comparative study was conducted on the effects of mono-*n*-butyl phthalate (MBP), di-*n*-butyl phthalate (DBP), MEHP and DEHP on energy coupling and electron transport activities of isolated rat liver mitochondria.

Materials and Methods

Mitochondria were isolated fresh daily from the livers of 250-g male rats of the CD strain, as described by Stancliff et al. (*8*). Protein concentrations were determined by the Lowry method (*9*) with the use of bovine serum albumin as a standard.

Respiratory rates were measured with a Clark-type oxygen electrode (*10*) in 2 ml of a medium consisting of 250 *mM* sucrose, 10 *mM* KCl, 10 *mM* Tris HCl (pH 7.6), 3 *mM* NaH_2PO_4, 5 *mM* $MgCl_2$, and 1.8 μg/ml rotenone. Mitochondrial samples (3 mg protein) were incubated for 2 min with the phthalate esters prior to the addition of substrate. Respiration was initiated by the addition of succinate (2.0 *mM* final concentration), and phosphorylation was initiated by the addition of 0.25 μmole of ADP. The control samples had the same final concentration of ethanol (0.24%) that resulted from the addition of the phthalate esters.

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Volume changes of mitochondria due to active K^+ uptake were measured (*11*) with a Beckman Acta III recording spectrophotometer at 540 nm. The concentrations of the components of the reaction medium were 100 *mM* sucrose, 28.6 *mM* potassium acetate, 5 *mM* Tris-acetate (pH 7.6), 1.2 μg/ml rotenone, 0.02 μg/ml valinomycin, and either 2.0 *mM* succinate, 2.0 *mM* ascorbate + 0.15 *mM* *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD), or 2.0 *mM* ATP. Mitochondrial samples (2 mg protein) were added to 3 ml of reaction medium and incubated with the phthalate esters for 2 min prior to the addition of the energy source.

Succinate cytochrome c reductase activity was measured (*12*) with a Beckman Acta III recording spectrophotometer at 550 nm in 3 ml of a medium that consisted of 20 *mM* potassium phosphate (pH 7.5), 2 *mM* KCN, and 0.15 *mM* cytochrome c. Mitochondrial samples (1 mg protein) were incubated in the reaction medium for 2 min to lyse the membranes, and then the phthalate ester was added and the sample was incubated for an additional 2 min. Reduction of cytochrome c was initiated by the addition of succinate (2 *mM* final concentration).

Reagent grade DBP was obtained from Fisher Scientific Co. Technical grade DEHP was obtained from the Hatco Chemical Division of W. R. Grace and Co. MEHP and MBP were synthesized by refluxing phthalic anhydride and 2-ethylhexanol or *n*-butanol in toluene and were then extracted as described by Albro et al. (*13*). Stock solutions of each of the phthalate esters were made in 95% ethanol.

Results

Energy-Dependent K^+ Uptake

In intact, coupled mitochondria, oxidation of added substrate or the hydrolysis of adenosine-5'-triphosphate (ATP) generates a potential across the inner membrane composed of a pH gradient plus an electrical gradient (the outside is positive with respect to the inside). The active transport of cations across the inner mitochondrial membrane is driven by the membrane electrical potential. K^+ is naturally impermeable to the inner mitochondrial membrane; however, the lipid-soluble ionophore, valinomycin, has a polar interior that delocalizes the cation charge so that the K^+ valinomycin complex can diffuse across the membrane. The energy-linked accumulation of K^+ in response to a charge gradient results in osmotic water flow which leads to mitochondrial swelling. The swelling results in greater light penetration through the mitochondrial suspension, and can be followed by monitoring changes in light absorption (*14*). We examined the effects of phthalate esters on energy-dependent K^+ uptake driven by the oxidation of two respiratory chain substrates, succinate and ascorbate-TMPD, and by the hydrolysis of ATP. We reasoned that, if there is a substrate specific effect, then K^+ transport would be affected differently with the different energy sources; and if the phthalate esters induce a nonspecific effect, then the energy-linked K^+ transport would be affected similarly with the three energy sources.

Table 1 presents a comparison of the effects of phthalate esters on energy-linked K^+ uptake in isolated rat liver mitochondria. These effects were determined by monitoring changes in absorbance at 540 nm. In the control, there is a rapid decrease in absorbance upon addition of valinomycin. A typical trace of change in absorbance at 540 nm of rat liver mitochondria due to succinate oxidation-induced K^+ uptake is shown in Figure 1. The maximum amplitude of swelling is reached about 30 sec after the addition of valinomycin. The mitochondria undergo a series of swellings and contractions until the oscillations become damped, largely due to a loss in synchrony and to membrane deterioration occurring during the swelling and contraction cycles (*11*). These volume changes do not occur if substrate oxidation is inhibited or if the membrane potential is collapsed by addition of an uncoupler.

In the presence of 1.0 *mM* MBP, swelling initiated with each of the three energy sources is slower than in the control and there is a loss of synchrony of swelling and contraction. Similar observations

Table 1. Effect of phthalate esters on energy-linked K^+ uptake in isolated rat liver mitochondria.[a]

	Energy source		
Condition	Succinate	Ascorbate-TMPD	ATP
MBP			
0.01*mM*	+ + + +	+ + + +	+ + + +
1.0*mM*	+ + +	+ + +	+ + +
DBP			
0.010*mM*	+ + + +	+ + + +	+ + +
0.025*mM*	+ + +	+ + +	+ +
0.1*mM*	–	–	–
1.0*mM*	–	–	–
MEHP			
0.1*mM*	+ + +	+ + +	+ +
1.0*mM*	–	+	+
DEHP			
0.1*mM*	+ + + +	+ + + +	+ + + +
1.0*mM*	+ + + +	+ + + +	+ + +

[a]Energy-linked swelling; (+ + + +) equivalent to the control; (+ + +) amplitude of swelling is equivalent to the control, but there is a loss of oscillations; (+ +) substantially decreased amplitude of swelling with no oscillations; (+) very slight uncoupler sensitive swelling; (–) no detectable energy-linked swelling.

have been made with uncouplers at low concentrations and are, therefore, indicative of a reduced energy supply (*14*). Reduction of the concentration of MBP to 0.1 *mM* results in absorbance changes that are nearly identical to that of the control. In the presence of 1.0 *mM* MEHP, swelling is abolished with succinate as the energy source; however, there is slight uncoupler-sensitive swelling with ascorbate–TMPD or ATP. Therefore, in addition to a common effect with each of the three energy sources, there is an additional effect of MEHP on succinate oxidation-linked swelling. Reduction of the concentration of MEHP to 0.1 *mM* resulted in increased active transport activity in each case. In the presence of 1.0 *mM* DEHP, the swelling traces with succinate and ascorbate–TMPD are similar to the control, indicating that there is no apparent deleterious effect. A slight alteration in ATP-supported K^+ uptake occurs at 1.0 *mM* DEHP. Since DEHP did not affect succinate or ascorbate-TMPD induced swelling, it is possible that the adenine nucleotide translocator or the ATPase is partially sensitive to DEHP. In the presence of 1.0 or 0.1 *mM* DBP, energy-linked K^+ transport initiated by oxidation of succinate or ascorbate–TMPD or by hydrolysis of ATP is totally abolished. In each case, energy-linked swelling (sensitive to uncoupler) is markedly improved when the concentration of DBP is reduced to 0.025 *mM*. At 0.010 *mM* DBP, the swelling traces are nearly identical to the control traces.

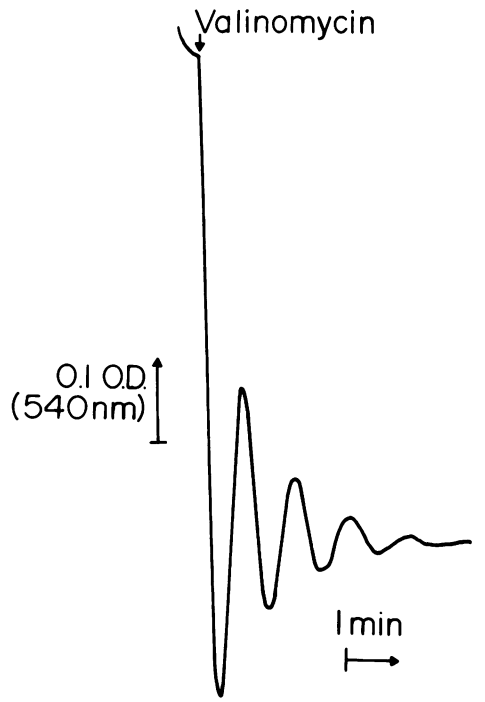

FIGURE 1. Oscillation of rat liver mitochondria due to K^+ transport linked to succinate oxidation.

Respiratory Control

Table 2 demonstrates the effects of phthalate esters on mitochondrial respiration with succinate as the oxidizable substrate. In coupled mitochondria, the proton electrochemical gradient that is generated during substrate oxidation suppresses electron transport to oxygen. The addition of ADP stimulates electron flow and oxygen consumption since this energized state is being utilized, in this case for ATP synthesis. The respiratory control ratio (RCR) is a measure of the dependence of the respiratory rate on the presence of ADP (state 3) divided by the rate after the expenditure of ADP (state 4). ADP stimulation represents the incre-

Table 2. Effect of phthalate esters on mitochondrial respiration.

Condition	RCR[a]	State 4 rate (% of control)	State 3 rate (% of control)	ADP stimulation (% of control)
Control	3.7	100[b]	100[c]	100[d]
MBP				
0.1*mM*	3.1	123	96	87
1.0*mM*	2.7	144	101	84
DBP				
0.1*mM*	1.4	285	89	33
1.0*mM*	1.1	297	82	9
MEHP				
0.1*mM*	1.6	167	71	34
1.0*mM*	–	109	22	0
DEHP				
0.1*mM*	3.6	103	96	93
1.0*mM*	3.6	101	98	96

[a]Respiratory control ratio.
[b]9.7 nmole oxygen/min-mg protein.
[c]35.9 nmole oxygen/min-mg protein.
[d]State 3 rate – State 4 rate; 26.2 nmole oxygen/min-mg protein.

ment in the respiratory rate after the addition of ADP. The respiratory control ratio, which is indicative of coupling between the oxidation and phosphorylation processes, is regarded as a good index of the integrity of mitochondrial structure and function. Generally, an increase in state 4 respiration leading to a loss of respiratory control is indicative of uncoupling by dissipation of the proton electrochemical gradient. Classical uncouplers (e.g., 2,4-dinitrophenol) act as proton ionophores promoting proton equilibration across the inner mitochondrial membrane. Inhibited respiratory rates are largely attributed to inhibition of substrate oxidation or ADP utilization. The changes in respiratory control due to the presence of the phthalate esters are similar to those reported by Takahashi (*6*). The greatest changes were observed (Table 2) with DBP and MEHP. There was a moderate effect with MBP, and there was essentially no change in the presence of DEHP.

The decrease in respiratory control due to MBP can be accounted for by the increase in state 4 respiration. DBP is an effective uncoupler of oxidative phosphorylation at concentrations of 1.0 and 0.1 *mM*. The effect is largely due to stimulation of state 4 respiration. The reduced rates in the presence of ADP (state 3 rate) indicate that there is also some inhibition of succinate oxidation or ADP utilization. As a result of these effects, there is nearly total loss in stimulation of respiration upon addition of ADP, i.e., there is essentially no phosphorylation of ADP. MEHP also promotes a loss of respiratory control. At 0.1 *mM* MEHP there is stimulation of state 4 respiration, and both 0.1 *mM* and 1.0 *mM* MEHP markedly decrease state 3 respiration rates. The latter effects are probably due to inhibition of succinate oxidation since the respiratory rates are not stimulated by the uncoupler, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), but are stimulated upon subsequent addition of ascorbate + TMPD (results not shown). Since ascorbate + TMPD donate electrons into the respiratory chain at cytochrome c, then inhibition of succinate oxidation by MEHP must occur between succinate dehydrogenase and cytochrome c. Thus, MEHP appears to act as an uncoupler of oxidative phosphorylation and an inhibitor of succinate oxidation. The combination of these effects limits or eliminates ATP synthesis, as indicated by the loss of ADP stimulation of state 4 respiration. DEHP had no apparent effect on mitochondrial respiration.

Succinate Cytochrome c Reductase Activity

Succinate cytochrome c reductase is a partial reaction of the electron transport chain, in which succinate serves as the electron donor and excess cytochrome c is provided as the electron acceptor (*12*). Potassium cyanide is added to block electron flow from cytochrome c to oxygen. The rate of cytochrome c reduction is measured spectrophotometrically at 550 nm. Since the catalytic site of succinate dehydrogenase is located on the inner surface of the inner mitochondrial membrane, the reaction was measured with osmotically lysed mitochondria in order to eliminate the carrier-mediated transport of succinate as the potentially rate-limiting step. MBP and DEHP had no effect on the activity, while DBP showed significant inhibition (Table 3). MEHP was the most potent inhibitor of the phthalate esters that were tested. In each case that inhibition was observed, the rate of cytochrome c reduction was increased maximally upon addition of NADH. Since both NADH and succinate cytochrome c reductase activities include the cytochrome b-c_1 region of the electron transport chain, then the inhibited rates shown in Table 3 can be attributed to inhibition of succinate dehydrogenase activity.

Discussion

The present studies show that phthalate esters affect isolated rat liver mitochondria by uncoupling energy-linked processes and by inhibiting succinate dehydrogenase activity. The phthalate esters that were examined vary in their potency in affecting these processes. MBP had a similar effect on the energy-linked K^+ uptake for the three different energy sources employed. It also stimulated succinate state 4 respiration rates. Based on these effects MBP can be considered to be a weak mitochondrial uncoupler. Classical uncouplers, such as FCCP, totally uncouple oxidative phosphorylation at micromolar concentrations (*15*). DBP was an inhibitor

Table 3. Effect of phthalate esters on succinate cytochrome c reductase activity of lysed mitochondria.

Condition	Succinate cytochrome c reductase activity, % of control
Control	100[a]
MBP	
0.1*mM*	106
1.0*mM*	95
DBP	
0.1*mM*	65
1.0*mM*	56
MEHP	
0.1*mM*	67
1.0*mM*	23
DEHP	
0.1*mM*	96
1.0*mM*	98

[a]118 nmole/min-mg protein.

of succinate dehydrogenase activity and was the most potent uncoupler tested. It interfered with K^+ uptake induced by all three energy sources; it stimulated by nearly threefold the succinate state 4 respiration rates; and it led to a nearly total loss of respiratory control. DBP is not a weak acid, as are the classical mitochondrial uncouplers that increase the passive permeability of the inner mitochondrial membrane to protons. Therefore, DBP probably uncouples energy-linked processes by partitioning in the mitochondrial membrane causing molecular conformational changes that render the membrane permeable to protons and other small ions. In support of this conclusion, Inouye et al. (*7*) found that exposure of isolated mitochondria to DBP results in release of intramitochondrial K^+ in the absence of valinomycin. In addition, Ohyama (*16*) reported that DBP stimulates the latent ATPase activity of whole mitochondria at concentrations that also induced nonenergy-linked mitochondrial swelling. These observations are consistent without contention that DBP alters the permeability properties of the inner mitochondrial membrane.

MEHP is also an uncoupler of mitochondrial energy-linked reactions. It impaired the energy-linked K^+ uptake initiated with three different energy sources, and it stimulated succinate state 4 respiration rates. There was total loss of respiratory control in the presence of 1.0 *mM* MEHP. Of the phthalate esters that were tested, MEHP was the most potent inhibitor of succinate dehydrogenase activity. We are currently investigating the nature of that inhibition. DEHP had no effect on liver mitochondrial activities other than weak inhibition of ATP-supported K^+ uptake.

Although these studies were conducted on isolated mitochondria, it is possible that the permeability properties of other cellular membranes are similarly altered by phthalate esters. The net effect of energy uncoupling and inhibition of substrate oxidation is a diminished chemical energy supply and altered regulation of ion transport in cells exposed to such compounds. These changes could modify energy-dependent cytoplasmic activities and the activities of ion-dependent enzymes. MEHP is a mitochondrial energy uncoupler and an inhibitor of succinate dehydrogenase activity. Since MEHP is a metabolite of DEHP, then it may play an important role in the hepatotoxic effects associated with exposure to DEHP.

It is interesting to note some of the similarities in effects of DEHP and clofibrate in rodents. Oral administration of either compound induces proliferation of peroxisomes (*3*, *17*, *18*), reduces plasma cholesterol and triglyceride levels (*17*, *19*) and results in the development of hepatocellular carcinomas (*2*, *20*). In addition, clofibrate ethyl ester has been shown to stimulate succinate oxidation rates of isolated mouse liver mitochondria, while the pharmacologically active metabolite formed by hydrolysis of the ester, sodium clofibrate, inhibits succinate oxidation (*21*). MEHP possesses an ester and a free carboxyl group, and depending on its concentration and the presence of ADP, MEHP stimulates or inhibits succinate oxidation rates of rat liver mitochondria. It is tempting to speculate that some of the effects of MEHP and clofibrate on rodent hepatocytes are a consequence of a primary effect on the mitochondria. The mitochondrial responses should occur before the other changes induced by these compounds are expressed. Therefore, we suggest that peroxisome proliferation may be a result of a cellular response to inhibition of mitochondrial succinate oxidation, and hypolipidemia may in part reflect decreased lipid biosynthesis due to reduced mitochondrial ATP synthesis or increased oxidation resulting from loss of respiratory control.

REFERENCES

1. Jaeger, R. J., and Rubin, R. J. Plasticizers from plastic devices; extraction, metabolism, and accumulation by biological systems. Science 170: 460-462 (1970).
2. National Toxicology Program: Bioassay of di(2-ethylhexyl) phthalate for possible carcinogenicity. National Toxicology Program, Research Triangle Park, N.C., 1981, DHHS Publication No. (NIH) 81-1773.
3. Lake, B. G., Gangolli, S. D., Grasso, P., and Lloyd, A. G. Studies on the hepatic effects of orally administered di-(2-ethylhexyl)phthalate in the rat. Toxicol. Appl. Pharmacol. 32: 355-367 (1975).
4. Strivastava, S. P., Seth, P. K., and Agarwal, D. K. Biochemical effects of di-2-ethylhexyl phthalate. Environ. Physiol. Biochem. 5: 178-183 (1975).
5. Ohyama, T. Effects of phthalate esters on the respiration of rat liver mitochondria. J. Biochem. 79: 153-158 (1976).
6. Takahashi, T. Biochemical studies on phthalic esters. II. Effects of phthalic esters on mitochondrial respiration of rat liver. Biochem. Pharmacol. 26: 19-24 (1977).
7. Inouye, B., Ogino, Y., Ishido, T., Ogata, M., and Utsumi, K. Effects of phthalate esters on mitochondrial oxidative phosphorylation in the rat. Toxicol. Appl. Pharmacol. 43: 189-198 (1978).
8. Stancliff, R. C., Williams, M. A., Utsumi, K., and Packer, L. Essential fatty acid deficiency and mitochondrial function. Arch. Biochem. Biophys. 131: 629-642 (1969).
9. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265-275 (1951).
10. Chance, B., and Williams, G. R. Respiratory enzymes in oxidative phosphorylation. III. The steady state. J. Biol. Chem. 217: 409-427 (1955).
11. Gooch, V. D., and Packer, L. Oscillatory states of mitochondria. Studies on the oscillatory mechanism of liver and heart mitochondria. Arch. Biochem. Biophys. 163: 759-768 (1974).
12. Sottocasa, G. L., Kuylenstierna, B., Ernster, L., and Bergstrand, A. An electron transport system associated with the outer membrane of liver mitochondria. A biochemical and morphological study. J. Cell Biol. 32: 415-438 (1967).
13. Albro, P. W., Thomas, R., and Fishbein, L. Metabolism of diethylhexyl phthalate by rats. Isolation and characteriza-

tion of the urinary metabolites. J. Chromatogr. 76: 321-330 (1973).
14. Gooch, V. D., and Packer, L. Oscillatory systems in mitochondria. Biochim. Biophys. Acta 346: 245-260 (1974).
15. Slater, E. C. Application of inhibitors and uncouplers for a study of oxidative phosphorylation. In: Methods in Enzymology, R. W. Estabrook and M. E. Pullman, Eds., Vol. 10, Academic Press, New York, 1967, pp. 48-57.
16. Ohyama, T. Effects of phthalate esters on the latent ATPase and swelling of mitochondria. J. Biochem. 82: 9-15 (1977).
17. Reddy, J. K., Moody, D. E., Azarnoff, D. L., and Rao, M. S. Di-(2-ethylhexyl) phthalate: an industrial plasticizer induces hypolipidemia and enhances hepatic catalase and carnitine acetyltransferase activities in rats and mice. Life Sci. 18: 941-946 (1976).
18. Hess, R., Stäubli, W., and Riess, W. Nature of the hepatomegalic effect produced by ethyl-chlorophenoxyisobutyrate in the rat. Nature 208: 856-858 (1965).
19. Best, M. M., and Duncan, C. H. Hypolipemia and hepatomegaly from ethyl chlorophenoxyisobutyrate (CPIB) in the rat. J. Lab. Clin. Med. 64: 634-642 (1964).
20. Reddy, J. K., and Qureshi, S. A. Tumorigenicity of the hypolipidaemic peroxisome proliferator ethyl-α-*p*-chlorophenoxyisobutyrate (clofibrate) in rats. Brit. J. Cancer 40: 476-482 (1979).
21. Yeh, R., and Kabra, J. J. Antagonistic effect of two forms of clofibrate on mitochondrial oxidation of succinate. Life Sci. 11 (Part II): 709-716 (1972).